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The Involvement of Interoceptive Chemosensory Activity in the Nervous Regulation of the Prothoracic Gland in a Moth, *Mamestra brassicae*

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ABSTRACT—Histidine-rich and trehalose-rich saline was applied to the isolated nervous system connected with the prothoracic gland (PTG) of the moth *Mamestra brassicae*. These salines produced a marked elevation of the afferent electrical activity in the nerve 4 to PTG and consequently a strong depression of the efferent activity. It is concluded that the afferent neurones in the nerve N4 are chemosensory and function as a sensor to monitor the histidine or trehalose concentration in the external medium. Further, the input from the afferent neurones must disinhibit the inhibitory function of the efferent neurones to PTG. The comparison these results with the determination of the change in sugar contents in the haemolymph during the larval development, suggests that it is the elevation of the trehalose concentration in the haemolymph during the progress of the development which, via the involvement of the afferents in N4, finally induces the accelerated ecdysteroid synthesis or release at PTG.

INTRODUCTION

In the preceding two papers [1, 2], it has been shown that the nerves to the PTG conduct both efferent and afferent electrical activity. From the measurements of efferent activity of nerves to PTG and the released ecdysteroids from PTG in the same isolated preparation, it was demonstrated that the efferent electrical activity of the nerve N4 inhibited the ecdysteroid synthesis or release at PTG.

The afferent electrical activity was shown to be generated at certain points of the nervous tract connected with PTG as well as at PTG itself [1]. An important suggestion as to its function was derived from the fact that the afferent electrical activity of the nerves was increased in response to some constituents in the Grace's medium [2] and net this activity appears to have an inhibitory influence on the generation of efferent activity.

The experiments reported here are intended to demonstrate chemoreceptive properties of the afferent neurones, and to show their inhibitory influence on the efferent neurone which in turn inhibits the PTG activity. To examine possible physiological functions of the chemoreceptive activity of the afferent neurone, the changes in chemical constituents of the haemolymph, such as sugars, were measured during the larval development.

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MATERIALS AND METHODS

Larvae of *Mamestra brassicae* were reared on artificial diet [3] at 23°±1°C under long day photoperiod (16L, 8D). Female larvae were used throghout this experiment.

Electrical activity of PTG innervating nerves was recorded as described in the previous paper [1]. The chemosensory region of the isolated nervous tract was stimulated by twice exchanging the external medium for the stimulant solution in the oil gap chamber. The change in electrical activities was recorded on tape recorder connected with the oscilloscope.

The chemosensory region of the nervous tract was prepared for histology using the modified

Bodian method.

For the determination of the change in sugar contents in the haemolymph during the larval development, 20 μl haemolymph was taken from a larva by making small cut on the proleg. The haemolymph was introduced into a trichloroacetic acid solution to remove proteins. The total sugar contents of the supernatant were determined by the anthrone-H_2SO_4 method standardized with glucose. The trehalose content in the total sugar was estimated by comparing the results obtained from two different determinations of the reducing sugar contents in the total sugar by the Somogyi-Nelson method and of the trehalose contents in the total sugar by reversed-phase high-performance liquid chromatography.

RESULTS

Selection of chemically stimulative solutions

It was found in the preceding study of this series [2] that afferent activity in the nerve N4 was induced in response to the Grace's medium. The chemical constituents of Grace's medium are summarized in the right column of Table 1 while the left column shows the constituents of the normal saline used in this experiment. The main difference in the constituents of Grace's from those of the normal saline is sucrose as disaccharide and various amino acids; another difference is in the osmotic concentrations.

The stimulative effects of hypertonic normal saline (370 m Osm) made up with excess glucose were tested and found to be practically negligible. Trehalose-rich, histidine-rich and K-rich salines were selected as the test solutions for chemical stimulation. The constituents of these solutions are shown in Table 1. Trehalose was selected as it is the main component of free sugars in the haemolymph of the preparation used here (See below). Histidine was selected as a common and representative amino acid in the haemolymph. The pH value of the histidine-rich saline was adjusted to 6.5 by mixing L-histidine and L-histidine-HCl solutions, in addition to the adjustment by the ordinary phosphate buffer.

Histology

Physiological techniques [1] have already shown that generation sites of afferent activity within the nerves to PTG are localized to specific regions of nerve branching. Whole mount histological preparations of the nerves to PTG were made to find the cell bodies of these afferent neurones. Figure 1 is the micrograph of the nervous tract to PTG as well as the locally enlarged one taken at the nerve branching just in front of PTG. Here, fairly large

Table 1. The chemical composition of various types of the saline used in this experiment. Concentrations of K-salts in the saline are shown as including the phosphate buffer (mM/l, pH 6.5)

	Glucose-rich normal saline	K-rich saline	Trehalose-rich saline	Histidine-rich saline	Grace's medium
K-salt	35	95	35	35	30
Na-salt	4	4	4	4	22
Ca-salt	4	4	4	4	7
Mg-salt	15	15	15	15	22
Mono-saccharide	160	40	0	0	4
Di-saccharide	0	0	160	0	80
Amino acid	0	0	0	160	70
Organic acid	0	0	0	0	9
Total milliosmolar concentration	300	300	300	300	366

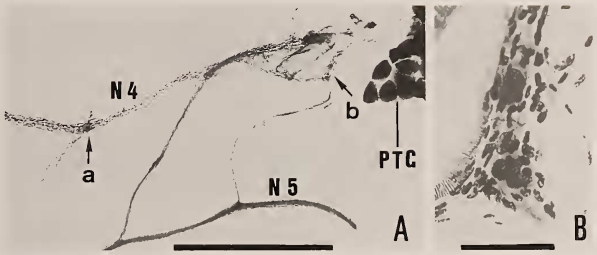

FIG. 1. Silver stained afferent neurone in the nervous tract N4 from prothoracic ganglion to PTG. For the size of cell body and nucleus, see text. A: Nervous tract to PTG; position of the cell body is shown by arrows (a, b). B: Enlargement of the position a in A. Scale bars in A and B are 500 μm and 50 μm respectively.

oval cell body can be seen among many glia cells, although its out line is not well distinct. The cell body was estimated from many preparations to be about 30 μm and 20 μm in apsidal and minor axes respectively. The nucleus was nearly a half the size of the cell body. This type of cell could be a chemosensory afferent neurone because, in many silver stained preparations of the nerve tract N4, it was the only clearly observable cell other than the glia cells seen. Distribution of the cell bodies of this type at the positions of each nerve branching of the nerve N4 was not perfectly consistent and consequently the number of cell bodies in each position ranged from zero, to one or two and some times three in different preparations. Total number of such cells in each preparation was about five. At least one cell body with the same shape could be seen at the surface of PTG tissues.

Stimulation of the chemosensory regions of the nerves to PTG

It has already been shown that the sites of generation of the afferent electrical activity are located both in the nerve N4 and in PTG itself [1]. The preparation used here to test chemical stimulation of these regions was the isolated ganglionic chain from brain to mesothoracic ganglion with the nerve N4. PTG was removed from the preparation because of the possibility that two chemosensory neuones with different physiological functions may exist within the nerves and PTG. Chemical stimulation of the PTG itself was not studied here.

In recordings of the electrical activity, the nearest region of the nerve N4 to the prothoracic ganglion was placed in position on the oil-gap. Both ganglionic chain and nervous tract were immersed separately in two normal saline pools of the oil-gap chamber for 1 hr before the chemical stimulation. This chamber was placed in a gas mixture containing 95% O_2 and 5% CO_2. Temperature was adjusted to $23^\circ \pm 1^\circ$C. For chemical stimulation, the normal saline in the one pool in which the nervous tract was immersed was replaced by a stimulant solution.

The effects of histidine-rich, trehalose-rich and K-rich salines are shown in Figures 2, 3 and 4, respectively. Each result is a representative example from several successful determinations.

Figure 2 shows the recordings of the electrical activity before and after the chemical stimulation as well as the wave forms of the efferent and afferent activity. The frequency histograms for both types of electrical activity recorded simultaneously from one nerve (N4) are also shown. Use of histidine-rich saline resulted in a remarkable augmentation of the afferent activity which appeared gradually and with long latency (lower histogram). This indicates that the afferent neurones in the nerves to PTG could be chemosensory in nature. Exact determination of the latency was difficult because the frequency of the electrical activity fluctuated during the period of determination even in the normal saline. Howev-

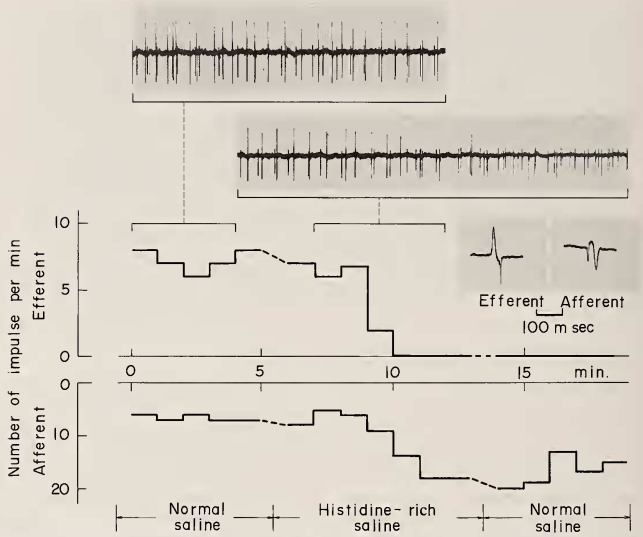

Fig. 2. Effects of histidine-rich saline applied to the nervous tract N4 from prothoracic ganglion to PTG. The period of application of various types of saline is shown in the lower part of the figure.

er, the extremely long latency observed in this sort of chemoreceptor is notable in comparison with the case of the chemical exteroceptors.

The upper histogram in Figure 2 also shows that the frequency of efferent activity decreased significantly and finally to zero as the afferent activity increased. The effects of histidine stimulation - high frequency of afferent activities and the following complete inhibition of efferent activity - continue usually for hours after a change back to normal saline. A serine-rich saline stimulated the afferent neurones similarly but to a lesser degree.

The stimulation effect of the trehalose-rich saline on the afferent neurones is shown by the frequency histograms in Fig. 3. The elevation of the afferent activity and resulting inhibition of the efferent activity were fundamentally the same as for histidine stimulation. Sucrose-rich saline (160 mM sucrose) was used for the chemical stimulation by another di-saccharide. This produced almost the same effects as trehalose-rich saline.

It was difficult to determine accurately the threshold value for inducing a clear change in frequency of the afferent activity because of large individual differences and unstable firing during the determination. However the general estimation obtained from many preparations was that the threshold concentration for inducing a significant effect within 5 minutes was about 30 mM for both histidine-rich and trehalose-rich salines. The possibility that 160 mM histidine or trehalose in the saline could induce a nonspecific irritation to the neurones can be excluded; the normal glucose-rich saline containing 160 mM glucose produced the minimal level of afferent activity and haemolymph of lepidopterous insects contains fairly large amount of trehalose and amino acids [4, 5].

Figure 4 shows the effects of stimulation by the K-rich saline. There was an immediate change in frequency of afferent as well as efferent activity at

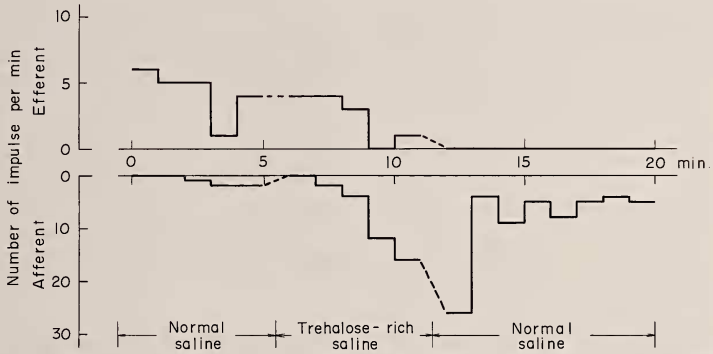

FIG. 3. Effects of trehalose-rich saline applied to the nervous tract N4 from prothoracic ganglion to PTG. Lower part of the figure drawn as in Fig. 2.

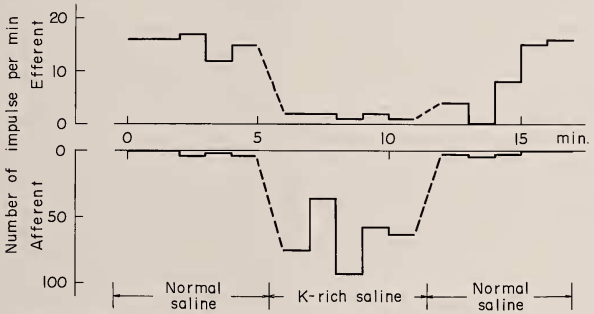

FIG. 4. Effects of K-rich saline applied to the nervous tract N4 from prothoracic ganglion to PTG. Lower part of the figure drawn as in Fig. 2.

the moment of application or removal of K-rich saline which clearly differed from the stimulation effect of histidine-rich or trehalose-rich salines. On removal of K-rich saline the efferent activity recovered from its inhibition within a few minutes after the fast return of the afferent activity to its normal level.

10^{-4} M juvenile hormone-II (JH-II) and 10^{-6} M or 10^{-8} M 20-hydroxyecdysone dissolved in the normal saline did not effect the afferent neurones (Fig. 5 shows JH saline). The histogram of the afferent activity in this preparation always showed zero level. The frequencies of the afferent activity in the normal saline were low in almost all preparation as shown in Figures 2–5, and the normal saline used in this experiment was the best medium for keeping the afferent activity low.

The results presented here lead to the conclusion that the afferent neurones in the nervous tract N4 are chemosensory and work as sensors to monitor the concentration of the chemical composition of the external medium. In particular they

Fig. 5. Effects of JH containing saline applied to the nervous tract N4 from prothoracic ganglion to PTG. Lower part of the figure drawn as in Fig. 2.

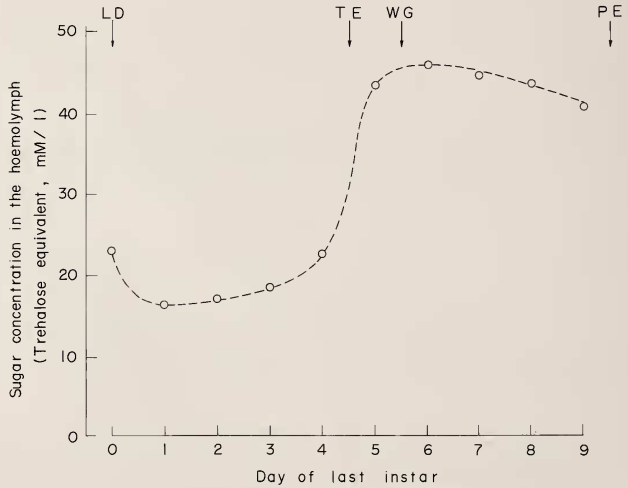

Fig. 6. The change of sugar content in the haemolymph during larval development. Sugar content was determined by the anthrone-H_2SO_4 method standardized with glucose but expressed in the figure by converting to the trehalose equivalents in mM/l. The following developmental stages are indicated by arrows: LE, larval ecdysis; TE, apperance of transparent epidermis; WG, wandering to the ground; PE, pupal ecdysis.

may sense the di-saccharides or amino acids. The input from the afferent neurones to the prothoracic ganglion inhibits the efferent activity to the PTG, and as this efferent activity is inhibitory, afferent activity effectively disinhibits PTG activity.

Changes of sugar contents in the haemolymph during larval development

One typical example of six series of determinations is shown in Figure 6. Sugar contents in the haemolymph can be seen to increase sharply from day 4 to day 5 during which period the transparent epidermis appears, i.e. above 30 mM/l, the minimum required *in vitro* to produce activation of the afferent neurones. The trehalose content in the total sugar was determined in the day 4 larvae by the methods described above, and estimated to be greater than 95% of the total sugar in the haemolymph.

DISCUSSION

In the experiments reported here, the afferent neurones in the nerves to PTG were shown to be chemosensory neurones sensitive to the chemical constituents of the haemolymph. So far similar visceral chemosensory neurones have been described only in vertebrates [6]. In the larva or the pharete-pupa of lepidopterous insects, several authors have demonstrated the feedback influence of the released hormones on the central programs for the various types of the developmental events [7~10]. The effect of the hormones in these cases appeared to be directly on the central ganglia. The existence of chemosensory afferent neurones in the nervous network which is supplied directly by the haemolymph is important, because changes in the specific chemical constituents in the haemolymph can be checked and conducted quickly to the central nervous system.

It was demonstrated here that the elevation of afferent neurone activity induced by the chemical stimulation was followed by a marked inhibition of efferent neurone activity. Such response was observed to continue for hours after the removal of stimulant solution. This fact suggests that the level of efferent activity appearing in the isolated preparations from various stages of last instar larvae [1] is not purely the outcome of the development of innate activity of the central nervous system, but the expression of an activity resulting from changes in specific chemical condition of the haemolymph and preserved for longer time after the isolation in the saline. Further analysis by intracellular recordings and the electronmicroscopic study may be necessary to understand precisely the physiological mechanism of these unique chemosensory neurones and this modulating influence to the central ganglia as described in this paper.

The results obtained here, as well as those shown in the previous papers [1, 2] that the function of efferent neurone on PTG is inhibitory, indicate that the marked elevation of the trehalose concentration in the haemolymph to some critical value during the development may be a cause of the accelerated PTG activity. This speculation is supported well by the fact that the estimated threshold concentration for the trehalose stimulation is about 30 mM in the sensitive preparations and the trehalose concentration in the haemolymph exceed this threshold value during the period from day 4 to day 5. However we must be careful in extraporating results from the isolated preparations to the normal situation.

Amino acid contents in the haemolymph were not determined. It seems premature to speculate on the overall function of the physiological responses induced by the individual amino acids. Although the biological functions of the amino acid sensitive neurones are not clear, the existence of the striking effects of amino acids, such as a histidine, on the afferent chemosensory neurones and of the consequent inhibition of the efferents to the PTG suggests they may act as regulators of PTG activity.

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REFERENCES

1 Okajima, A. and Watanabe, M. (1989) Electrophysiological identification of neuronal pathways to the prothoracic gland and the change in electrical activities of the prothoracic gland innervating neurones during larval development of a moth, *Mamestra brassicae*. Zool. Sci., 6: 459–468.

2 Okajima, A. and Kumagai, K. (1989) The inhibitory control of prothoracic gland activity by the neurosecretory neurones in a moth, *Mamestra brassicae*. Zool. Sci., 6: 851–858.

3 Agui, N., Ogura, N. and Okawara, M. (1978) Rear-

ing of cabbage armyworm, *Mamestra brassicae* L. (Lepidoptera: Noctuidae) and some lepidopterous larvae on artificial diet. Appl. Ent. Zool., **19**: 91–96.

4 Wyatt, G. R. (1961) The biochemistry of insect hemolymph. Ann. Rev. Entmol., **6**: 75–102.

5 Florkin, M. and Jeuniaux, C. (1974) Haemolymph: Composition. In "Physiology of Insecta, vol. 5". Ed. by M. Rockstein, Academic Press, New York, pp. 255–307.

6 Mei, N. (1983) Sensory structures in the viscera. In "Progress in sensory physiology, vol. 4". Ed. by D. Ottoson, Springer-Verlag, Berlin, pp. 1–42.

7 Gilbert, L. I., Bollenbacher, W. E., Agui, N., Granger, N. A., Sedlak, B. J., Gibbs, D. and Buys, C. M. (1984) The prothoracicotropes: Source of the prothoracicotropic hormone. Amer. Zool., **21**: 641–653.

8 Dominick, O. S. and Truman, J. W. (1984) The physiology of wandering behaviour in *Manduca sexta*. I. Temporal organization and the influence of the internal and external environments J. Exp. Biol., **110**: 35–51.

9 Fujishita, M., Ohnishi, E. and Ishizaki, H. (1982) The role of ecdysteroids in the determination of gut-purge timing in the saturniid, *Samia synthia ricini*. J. Insect Physiol., **28**: 961–967.

10 Truman, J. W. (1978) Hormonal release of stereotyped motor programs from the isolated nervous system of the cecropia silkmoth. J. Exp. Biol., **74**: 151–173.